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PRINCIPAL INVESTIGATOR: Guillermo A. Calero, M.D., Ph.D.
Richard A. Cerione, Ph.D.
Jon Clardy, Ph.D.

CONTRACTING ORGANIZATION: Cornell University
Ithaca, New York 14853-2801

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Guillermo A. Calero, M.D., Ph.D.
Richard A. Cerione, Ph.D.
Jon Clardy, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Cornell University
Ithaca, New York 14853-2801

E-Mail: gac9@cornell.edu

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**13. ABSTRACT** *(Maximum 200 Words)*

This project involves structural studies of a nuclear target for the EGF receptor, and the elated Neu/ErbB2 tyrosine kinase, named the CBC for RNA-capped binding protein complex. The CBC consists of two subunits, CBP20 (Mr 18 kDa) and CBP80 (Mr 90 Kda), and undergoes a growth factor (EGF, heregulin)-dependent binding of RNAs transcribed by the RNA polymerase II at a 5′ cap structure that consists of a guanosine residue methylated at the N7 position. This represents a first key step in the cap-dependent splicing of precursor messenger RNA (mRNA) and in the mucleocytoplasmic transport of U snRNAs which are necessary for the formation of the spliceosome complexes. While, EGF stimulates CBC activity, it is most strongly stimulated by heregulin, an activator of the Neu/ErbB2 tyrosine kinase, and appears to be constitutive in breast cancer where Neu/ErbB2 expression is high. Thus, we believe that the CBC represents an exciting nuclear target for receptor tyrosine kinases, linking growth factor-dependent gene expression to RNA processing. We have solved the atomic structure of the CBC in complex with m7GpppG at 2.2 Å. The atomic structure of this triple complex represents the second eukaryotic cap binding structure solved to date and reveals interesting aspects of capped RNA binding and regulation.

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## INTRODUCTION

Normal and malignant human mammary epithelial cells are able to synthesize and to respond to various different, locally acting growth factors through specific receptors. Among these are the type 1 family of growth factor receptors, which consist of the epidermal growth factor receptor (EGF-R), ErbB2/Neu, ErbB3, and ErbB4 (1-6). They are required for normal mammary development and lactation and are aberrantly expressed in approximately 40% of breast carcinomas. Indeed, in human breast cancer cases the prognosis of a patient is inversely correlated with the over expression and/or amplification of this receptor family. The physiological regulatory ligand for ErbB2/Neu has been shown to be heregulin (7-9). Interaction of heregulin with the ErbB3 induces a heterodimerization between ErbB2 and ErbB3, which results in the transphosphorylation and activation of the ErbB2 receptor. Phosphorylation of this receptor initiates signaling cascades, which in turn can impact upon cell function, growth and division.

One way to regulate protein expression in the cell is achieved by controlling mRNA concentration. Cytoplasmic mRNA levels represent a balance between transcription, splicing and nuclear export on the one hand and mRNA degradation on the other. The balance between these two processes represents a major control point in gene expression. Recent studies have shown that post-transcriptional mechanisms regulate protein expression in certain cell lines. For example, down-regulation of the proto-oncogene c-myc mRNA during differentiation of C2C12 myoblasts to myotubes is mediated by a cytoplasmic mRNA turnover event rather than a nuclear processing event (10). More importantly, Balmer et al (11), have show that the EGF-induced up-regulation of EGF-R mRNA in two human breast cancer cell lines that over-express EGF-R (MDA-MB-468 and BT-20) is accompanied by stabilization (>2-fold) of EGF-R mRNA. They showed that the EGF-R mRNA contains a novel complex AU-rich 260-nt cis-acting destabilizing element in the 3'-UTR that is bound by specific and EGF-regulated trans-acting factors.

Wilson *et al.* (12-13) have identified a novel nuclear target for heregulin signaling which responds to the growth factor treatment of cells with an increase ability to be labeled with GTP. They identified this target as the 20-kDa subunit of the nuclear cap binding complex (CBC) and demonstrated that the CBC is stimulated to bind to capped RNAs in response to heregulin. Based on these observations Wilson *et al* suggested that heregulin could impact upon cell growth by modulating gene expression at the level of RNA processing via the CBC. They further suggested that in a situation where the heregulin signal is constitutive, the active CBC could affect gene expression by amplifying the rate of RNA processing, and thus contribute to unregulated cell growth and division.

The CBC is comprised of a stable heterodimer between an 18-kDa subunit and a 90-kDa subunit (CBP20 and CBP80 respectively). Biochemical and genetic experiments have shown important roles for CBC in mRNA physiology including splicing (14-17), nuclear export (18-19), 3' end processing (20), translation initiation (21), nonsense mediated decay (NMD) (22) and mRNA degradation (23). Due to its active role in mRNA stability the CBC could play a role in protein expression, Regulation of CBC by growth factor signaling may represent in turn a mechanism to change cytosolic or nuclear protein levels.

We have solved the structure of the CBC at 2.2 Å resolution by molecular replacement (using as a model a partially proteolyzed CBC) and the phases from a Kr MAD dataset. Mazza et al (24) had previously published the structure of CBP80 and a proteolyzed fragment of CBP20 (residues 38-116). Due to proteolysis their structure could not answer several important questions such as how the cap binds to CBP20 and how CBP80 increases the affinity of CBP20 for the cap structure. The atomic structure reported here comprises CBP80, 95% of CBP20 (residues 7-153) and the cap structure analog m7GpppG.

## BODY

### 1. Experimental Procedures

Purification and crystallization protocols for CBC were outlined in the previous report.

***Solving the phase problem***

Table 1 summarizes crystallographic and phasing data

*Molecular Replacement*

Model: CBP80 from Mazza et al. (24) and a fragment of CBP20
Correlation Coefficient: 24.7
Rfactor (%) 42

*Refinement*

Resolution (Å) 50-2.2
No of reflections (test set) 51677 (2571)
Rcrys/Rfree (%) 21.7/25.7
Number of atoms 7240
Water Molecules 391
Average B Factor ($Å^2$) 40.2
Rms. bonds (Å) 0.007
Rms. angles (°) 1.6
Percent of residues in most favored regions 90.0%
Percent of residues in additionally allowed regions 9.4 %

### 2. Results

*The CBC*

Figure 1 illustrates the triple complex formed by CBP20, CBP80 and the cap analog m7GpppG. The atomic structure of CBP80 presented here is identical to the structure solved by Mazza et al. (24). CBP80 is all α helical and consists of three domains connected by two long linkers. Domains 1 (D1) and 3 (D3) are packed against domain 2 (D2) that constitutes the core of the protein. The N-terminal domain (D1) is structurally similar to the middle domain of eIF4G which plays a regulatory role in RNA translation and protein synthesis. Interestingly linker 1 connecting D1 and D2 contains a surface exposed proline rich sequence 268-PPFTP**P**PH-277 (**P**, conserved residue). Further biochemical experiments are needed to test possible interactions of this proline rich region with SH3 domains. The detailed structure of CBP80 was previously reported by Mazza et al. (24) and will not be the subject of this report.

*Overall Fold of CBP20*

The overall fold of CBP20 (Figure 2) is that of a classical ribonucleotide binding domain (RNP) and consists of four anti-parallel β-sheets packed against two α-helices. β3 (residues 81-88) and β1 (residues 41-46) form the RNP1 and RNP2 motifs respectively. Other important regions include the N-terminal region that interacts with CBP80, a loop (loop 3) between β2 and β3 and a C-terminal α-helix (αC) that participate in RNA binding. Modifications to the classical RNP fold include a long N-terminus and two insertions consisting of two sets of small anti-parallel β strands, the first between β4 and αC and the second at the C-terminus.

*Cap Binding to CBP20*

The methylated guanidine ring of the cap analog m7GpppG binds inside a cavity in CBP20 (Figure 3). Recognition and binding of the cap structure requires the following: 1) Enhanced stacking interactions between the electron deficient m7 guanine ring and two electron rich aromatic residues (Y20 and Y43) (Figure 4). 2) A "planar" network of electrostatic interactions (hydrogen bonds and salt bridges) between the guanosine base and a semicircular loop (residues 112-133)(Figure 5). Mutagenesis studies in this region are needed to establish the contribution of each residue to cap recognition and binding.

The mechanisms for the stabilization of the non-methylated guanosine base of m7GpppG is similar to other reported RNP proteins and is achieved through hydrophobic interactions with V134, hydrogen bonding with main chain atoms (amide nitrogen of R127 and carbonyl oxygen of R129) and Y138.

*Role of CBP80 in cap binding stabilization*

Izaurralde et al. (15) had shown previously that binding of the cap analog to CBC is inherently more stable than binding of the cap to CBP20 alone. This question was still outstanding after the publication of the structure of CBC by Mazza et al. (24). The N-terminus of CBP20 (residues 6-14) makes extensive hydrophobic and ionic interactions with residues from four α-helices (residues 31-39, 67-71, 323-335) in CBP80 (Figure 6). By stabilizing the N-terminus of CBP20 and the residues close to Tyr20 (stacking interactions with the m7-guanine ring) CBP80 increases the affinity of CBP20 for the cap structure.

*Phosphorylation of Threonine 79*

We have observed electron density for a possible phosphorylation site at Thr79 (loop 3). Comparison of this region between our structure and the structure of Mazza et al. (24) reveals a small change involving Tyr49 and Phe50 (figure 7) that could lead to stabilization of the C-terminus of CBP20 (which participates in RNA binding). At the moment this is just a speculation. Further experiments are needed to corroborate this finding and to establish the possible role of phosphorylation (if any) in RNA binding to CBP20.

*Possible Loop3 movements upon cap RNA binding*

Further comparisons between the bound structure reported here with the unbound structure reported by Mazza et al. (24) show potential rearrangements in loop 3. Upon RNA binding, Gly72 could function as a pivot point for loop 3 movements, since in the unbound structure Leu73 localizes to the cap-binding site (Figure 8). Most loop 3 regions (from sequences whose secondary structure and topology are known) vary in sequence and number of residues and can assume a wide range of conformations that could be important to RNA binding. We have recently obtained crystals of CBC without the cap analog. Solving this structure should provide important insights into the specific structural changes associated with cap binding.

*A possible RNA binding region in CBP20*

In RNP proteins, loops 1 and 3 and the C-terminal αC have been shown to participate in mRNA binding (25-26) A positively charged groove (Figure 9, blue arrows) is present on the surface of CBP20 just inferior to the non-methylated guanosine residue. This groove is formed from residues in loop 1 (floor), loop 3 and the C-terminal domain (lateral wall) in CBP20 and residues from CBP80 (opposing lateral wall and pocket). We have found a tubular electron density inside this pocket in CBP80 and we modeled it with a molecule of polyethylene glycol 400 (PEG400).

*Is CBC a decapping enzyme?*

Residues close to the ribose and the phosphates (Gln27, Gln133, and Arg127) in the cap-binding cavity present an architecture that could resemble the Ras/Ras-Gap complex (including Gln61 from Ras and Arg789 from RasGap). This finding raises the possibility that under certain circumstances (e.g. binding to another partner),

CBP20 could have decapping activity by hydrolyzing the gamma phosphate of m7Gppp. Interestingly, the yeast decapping enzyme, Dcp1p (25-kDa) shares 15.2% identity with CBP20 and has among its conserved residues, Tyr68 (Tyr43 in CBP20) which participates in cap binding (see above).

## KEY RESEARCH ACCOMPLISHMENTS

- Expression of CBC in SF9 cells.
- Purification and crystallization of CBC.
- Solution of the atomic structure of CBC in complex with m7GpppG at 2.2 Å resolution.
- Refinement of the CBC structure with a final $R_{free}/R_{factor}$ of 25.7/21.7 and good geometry.
- Crystallization of the unbound complex

## REPORTABLE OUTCOMES

- Manuscript in preparation:

ATOMIC STRUCTURE OF THE NUCLEAR CAP BINDING PROTEIN (CBP20) IN COMPLEX WITH CAP BINDING PROTEIN 80 (CBP80) AND THE CAP ANALOG m7GpppG.
G.A. Calero, K.F. Wilson, J.L. Rios, T.K. Ly, R.A. Cerione and J.C.Clardy.

## CONCLUSIONS

We have solved the structure of the triple complex between CBP20, CBP80 and the cap analog m7GpppG at 2.2 Å resolution. The atomic structure of this triple complex represents the second eukaryotic cap binding structure solved to date, the first being eukaryotic initiation factor 4E (eIF4E), and includes key structural aspects that were not found in the partial structure for the CBC published by Mazza et al (24).

The fold of CBP20 conforms to a classical RNP fold and differs significantly from the other two cap binding proteins, eIF4E (8 stranded antiparallel β sheets packed against 3 long helices)(27) and the vaccinia virus cap binding protein VP39 (7 stranded β sheets surrounded by 5 parallel helices) (28-29).

Binding of the capped RNA to CBP20 is slightly different from cap binding by eIF4E and VP39. Table 2 illustrates some of these differences (29). The low B-factors observed (35-40 $Å^2$) and the excellent electron density for the methylated guanosine base could indicate a tight interaction with CBP20.

An important aspect of the structure of the CBC is the possible stabilization of cap binding by CBP80. This is a novel finding for cap binding proteins and could represent an important regulatory mechanism. For example, we have seen that binding of importin-α to the N-terminal nuclear localization signal (NLS) of CBP80 increases cap affinity (see previous report). The structure of the CBC shows that residues in the N-terminus of CBP80 participate in cap stabilization, therefore interactions of importin-α with CBP80 could lead to increased cap-binding affinity. On the other hand, disruption of the CBC complex could lead to the release of capped RNA.

Two more aspects of the structure deserve comment. First, the strong electron density associated with T79 raises the possibility that this residue could be phosphorylated. Second, the supposition that CBP20 could be a decapping enzyme based on the structural similarities between the GTP binding site within the Ras/Ras-Gap and the cap-binding site within CBP20. At the moment, these are just speculations raised by structural findings and represent a clear example of how structure can drive biochemistry. Experiments are being

conducted (decapping assays) to investigate the role of the CBC in decapping using the yeast-decapping enzyme Dcp1p as a control.

The structure of CBP20 in complex with CBP80 and m7GpppG gives us new clues regarding how CBP20 a RNP protein, interacts with capped RNA and how CBP80 regulates this interaction. We know that CBP80 is needed for nuclear export of the CBC but the fact that CBP80 is significantly larger than CBP20 raises the possibility that CBP80 could also function as a scaffold for other proteins that could interact with CBP20 or the bound capped mRNA. The interaction of CBP20-CBP80 is reminiscent of the interaction between eIF4E and eIF4G (domain similarity with CBP80). In this case eIF4G works as a binding platform for other proteins such as eIF4E, eIF4A (a helicase) and the poly-A binding protein (30-32) among others.

New biochemical and structural studies are needed to fully understand the role of CBC in mRNA stability and how this can be influenced by signaling events via EGF-R or Neu/Erb2. During the last six months of my fellowship, I will concentrate on trying to obtain the structure of the unbound CBP20-CBP80 and to improve the crystals for the CBC-importin-α complex.

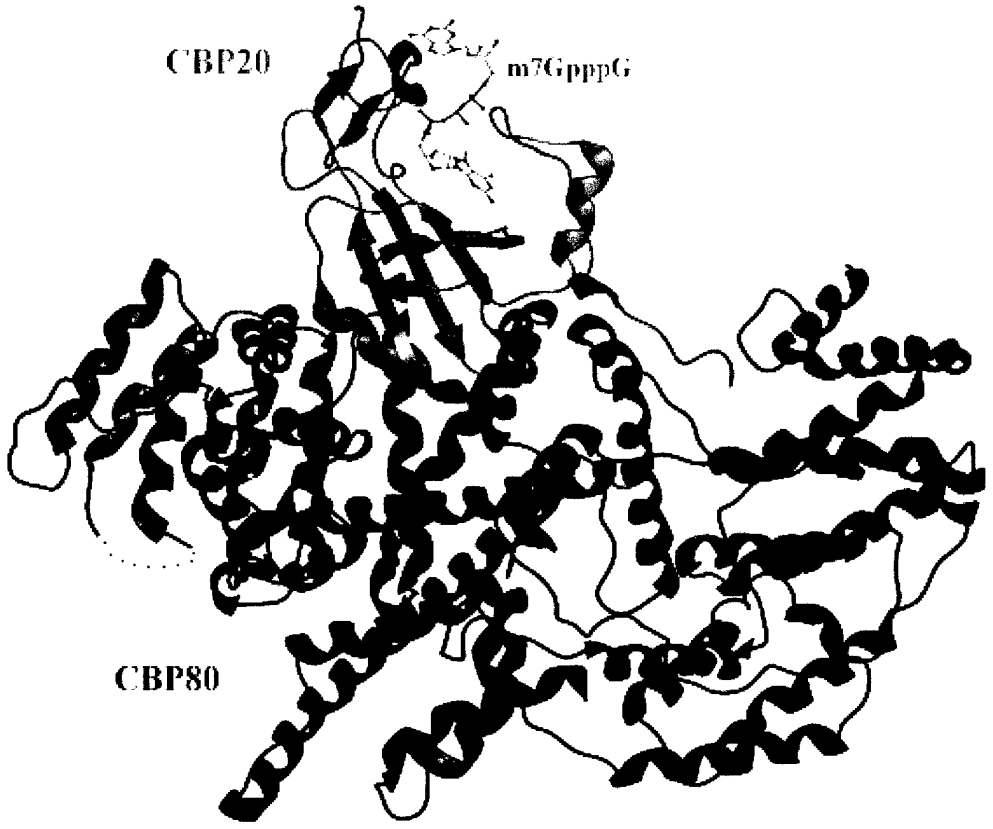



**Figure 1**

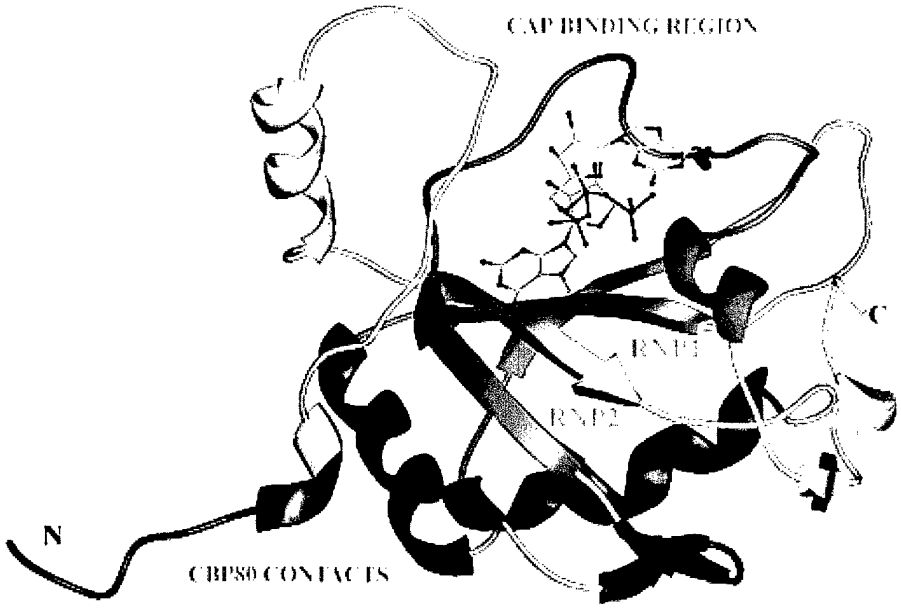



**Figure 2**

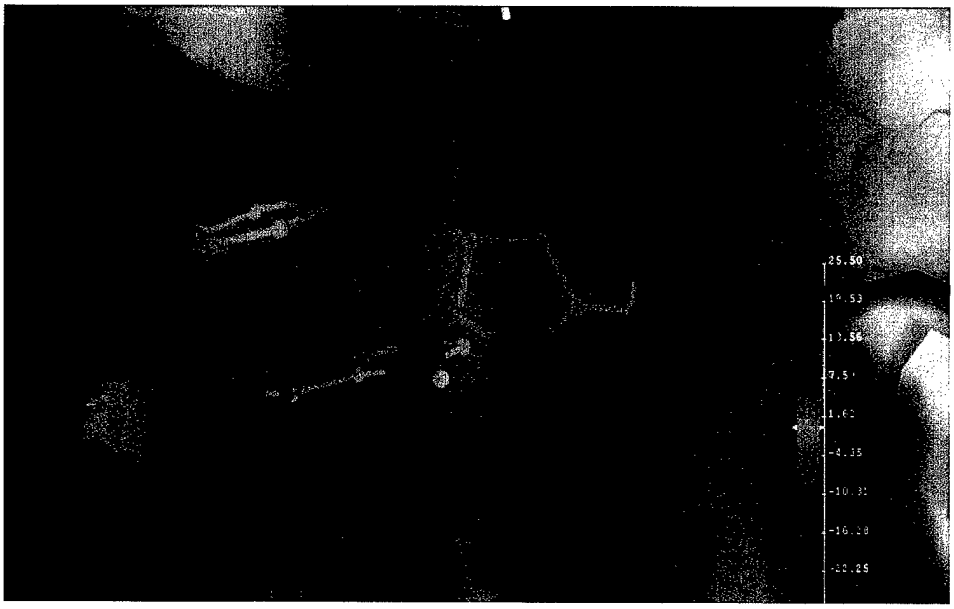


Figure 3

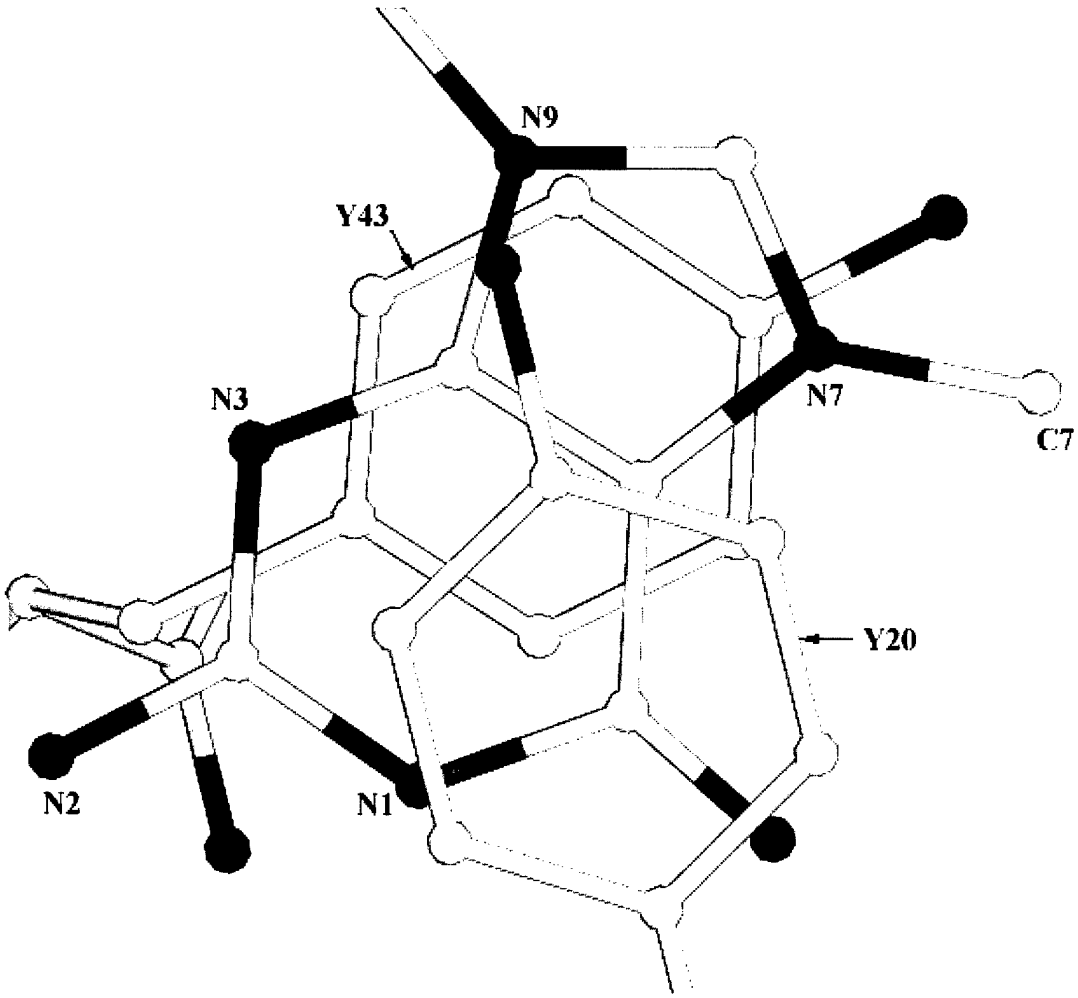
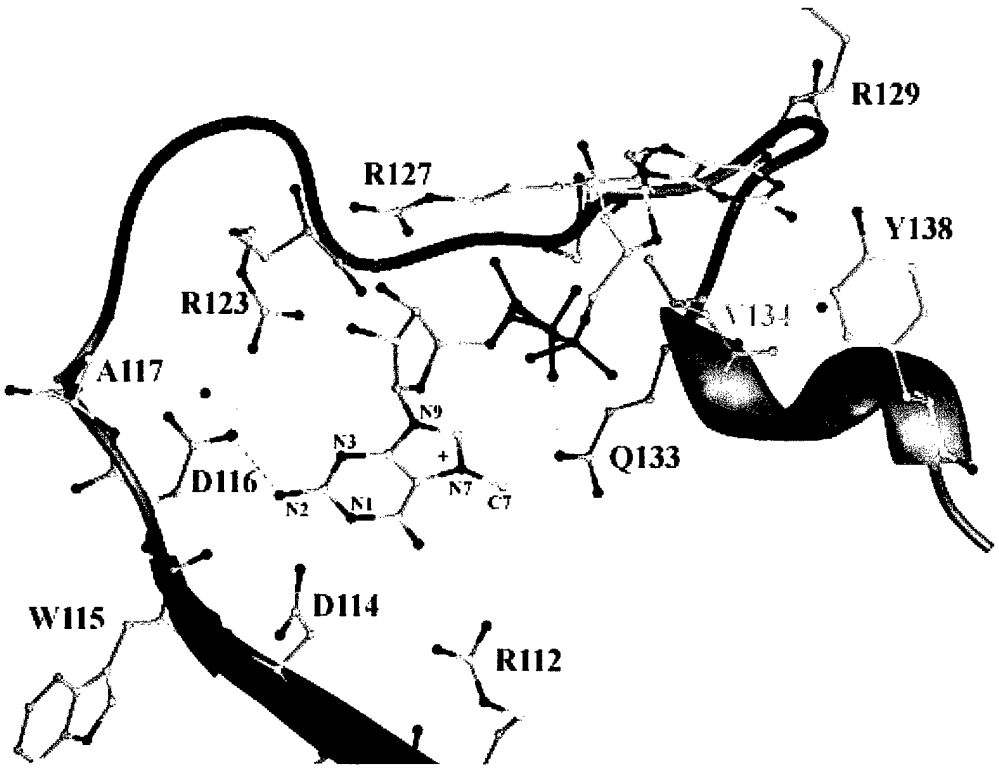



Figure 4

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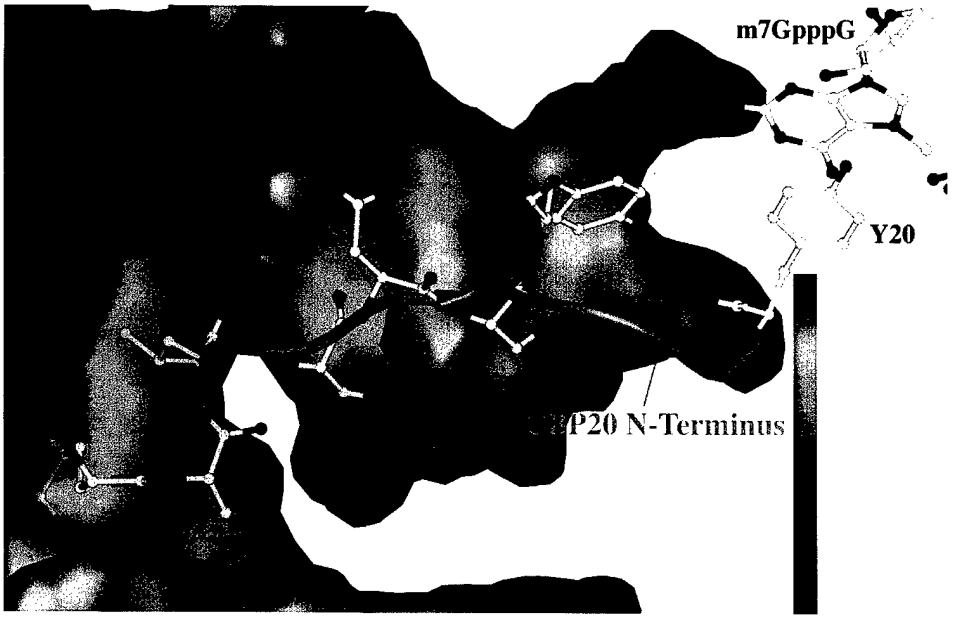


**Figure 6**

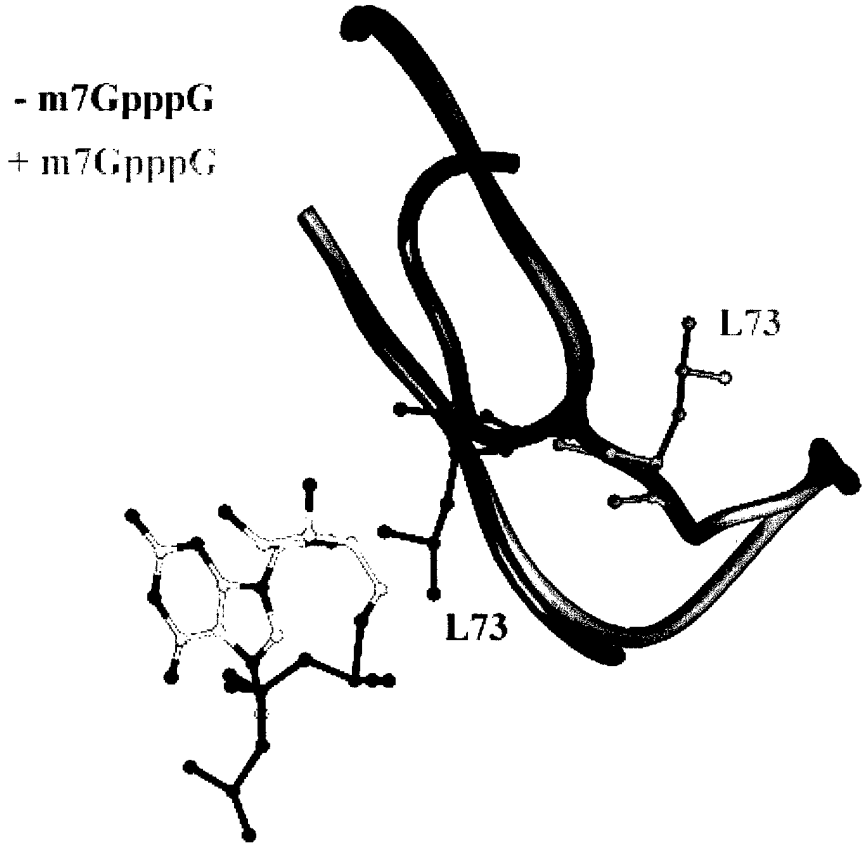


**Figure 7**

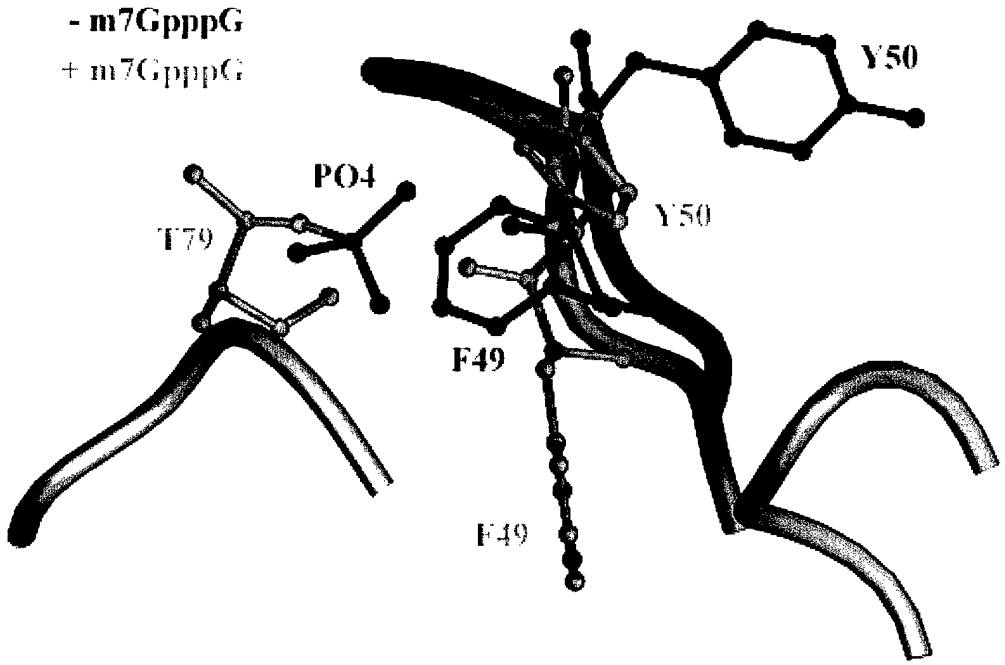


**Figure 8**

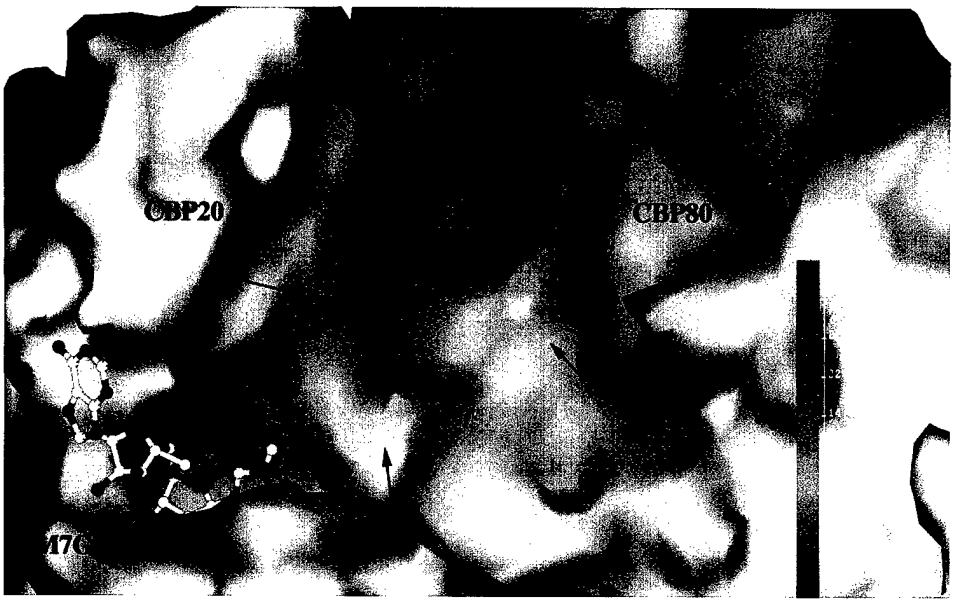


**Figure 9**

| | *Native* | *Osmium* | *Kr $\lambda_1$ (edge) 1 site* | | *Kr $\lambda_2$ (peak) 1 site* | | *Kr $\lambda_3$ (remote) 1 site* | |
|---|---|---|---|---|---|---|---|---|
| **Resolution (Å)** | 40-2.35 | 40-2.15 | 40-2.65 | | 40-2.65 | | 40-2.65 | |
| **Wavelength (Å)** | 0.9347 | 1.00 | 0.8651 | | 0.8655 | | 0.850 | |
| **Rmerge (%)** | 6.0 | 5.8 | 7.5 | | 7.6 | | 7.7 | |
| **I/σI** | 3.4 | 3.4 | 3.1 | | 3.1 | | 3.1 | |
| **Reflections** | 54292 | 70263 | 34246 | | 34259 | | 34160 | |
| **Redundancy** | 5.6 | | 12.8 | | 12.7 | | 12.4 | |
| **Completeness** | 99% | 99% | 95% | | 95% | | 95% | |
| **Rcullis (%)** | | 0.87 | Iso | Ano | Iso | Ano | Iso | Ano |
| | | | NA | 0.92 | 0.5 | 0.92 | 0.51 | 0.91 |
| **Phasing Power** | | Iso | Iso | Ano | Iso | Ano | Iso | Ano |
| | | 1.1 | NA | 0.98 | 1.24 | 0.99 | 1.39 | 1.04 |

**Table 1: Data collection statistics**

| | *Cavity Size* | *Second. Structure* | *Pi-Pi stacking residues* | *Hydrogen bonds* | *m7 Van der Waals contacts* | *PO4 Contacts* | *Ribose contacts* |
|---|---|---|---|---|---|---|---|
| **CBC** | +++ | β strand, | Y20 | 8 | none | Q133, | 2 R123 |
| | | loop | Y43 | | | R127 | 1 R123 |
| **IF4E** | ++ | Loop | W 56 | 3 | W166 | R157, | VdW |
| | | Loop | W102 | | | L162 | W56 |
| **VP39** | + | Helix | Y22 | 5 | Y204 | R177 | None |
| | | Loop | F180 | | | | |

**Table 2**

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